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14. ABSTRACT

The Ron receptor tyrosine kinase is over-expressed and over-activated in a cohort of human cancers, with the most compelling data yet found in breast cancer. Specifically, Ron is overexpressed in approximately 50% of human breast cancers, and has been shown to be an independent predictor of both metastases and poor prognosis in women with this disease. While Ron overexpression appears to be an important factor in human breast cancer growth and metastasis, a significant gap exists in our knowledge about the signaling pathways that Ron activates in breast tumors, and about the importance of these pathways with respect to overall tumor growth and metastatic dissemination. Our laboratories have shown that mammary tumors from mice overexpressing Ron selectively in the mammary epithelium exhibit increased levels of the DEK proto-oncogene. In addition, we also show that ligand-induced Ron activation in human and murine breast cancer cell lines induces the accumulation of DEK protein. This accumulation of DEK is significant as DEK overexpression in breast cancer cell lines leads to increases in cell growth and migration while DEK depletion in breast cancer cells leads to dramatic reductions in cell growth and migration. Moreover, we also show that DEK deficient cells are more susceptible to DNA damage. Based on these data, our goal is to test the *hypothesis that Ron-mediated DEK upregulation contributes functionally to breast cancer development, dissemination and resistance to clastogenic therapies and that targeting the Ron-DEK signaling axis may represent an important new therapeutic option for the treatment of breast cancer.* To test this hypothesis, **two Specific Aims** were proposed. In Aim 1, we will determine the requirement of DEK in Ron overexpressing breast cancers utilizing a combination of DEK loss of function and Ron transgenic overexpression. Aim 2 will examine the therapeutic utility of targeting Ron and DEK on beta-catenin activation and breast cancer growth. The proposed studies are uniquely innovative in many aspects. First, the experiments involve a new murine model of aggressive breast cancer that was developed in the Waltz laboratory, which mimics Ron overexpression observed in human patients. Second, we are using a new DEK knockout mouse model that was characterized and recently reported by the Wells laboratory. Third, the role of DEK in Ron-driven breast cancer, alone or in combination with chemotherapy has not been tested. Fourth, directly assessing the involvement of the Ron-DEK axis in breast cancer is a novel idea as it is unknown if DEK loss will reduce local tumor growth and/or the incidence of metastasis in a relevant *in vivo* system of breast tumorigenesis. Thus, we feel the new connection of the Ron-DEK signaling pathway and Ron/DEK as therapeutic targets represents a highly innovative area of study which may have an enormous impact on future diagnosis and treatment of patients with breast cancer.

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INTRODUCTION

The overall goal of our study to is test the hypothesis that Ron-mediated DEK upregulation contributes functionally to breast cancer development, dissemination and resistance to clastogenic therapies and that targeting the Ron-DEK signaling axis may represent an important new therapeutic option for the treatment of breast cancer.

BODY

To meet the goals of this study, two Tasks were outlined in the statement of work.

Task 1: Determine the functional significance of DEK expression in Ron-driven breast tumorigenesis and tumor response to chemotherapy.

Task 2: Examine the therapeutic utility of targeting Ron and DEK on beta-catenin activation and breast cancer growth.

Task 1 was split into two main objectives. The first objective was to define the role of Dek in Ron-driven tumorigenesis, which has been a focal point of our initial work. To support this objective, preliminary data in **Figure 1** depicts enhanced Dek expression observed in mammary tumors derived from MMTV-Ron expressing mice compared to Dek protein levels in normal mammary glands (**Figure 1A**). MMTV-Ron mice are a well-characterized transgenic model wherein wild type Ron is overexpressed selectively in the mammary epithelium by the mouse mammary tumor virus promoter (MMTV) (1). To more specifically examine Dek protein levels in the mammary glands of MMTV-Ron mice, mammary epithelial cells were isolated from wild type mice or from pre-neoplastic glands of MMTV-Ron mice. Lysates were generated and examined by western blot analysis for Dek expression. As shown in **Figure 1B**, Dek protein levels are elevated in the epithelium of MMTV-Ron mice. Moreover, Dek expression is also elevated *ex vivo* in the mammary tumor cell line derived from MMTV-Ron mice, termed R7 cells, in response to exogenous administration of the Ron ligand, hepatocyte growth factor-like protein (HGFL, **Figure 1C&D**). These data support the contention that Dek may be a key oncogenic target downstream of Ron signaling.

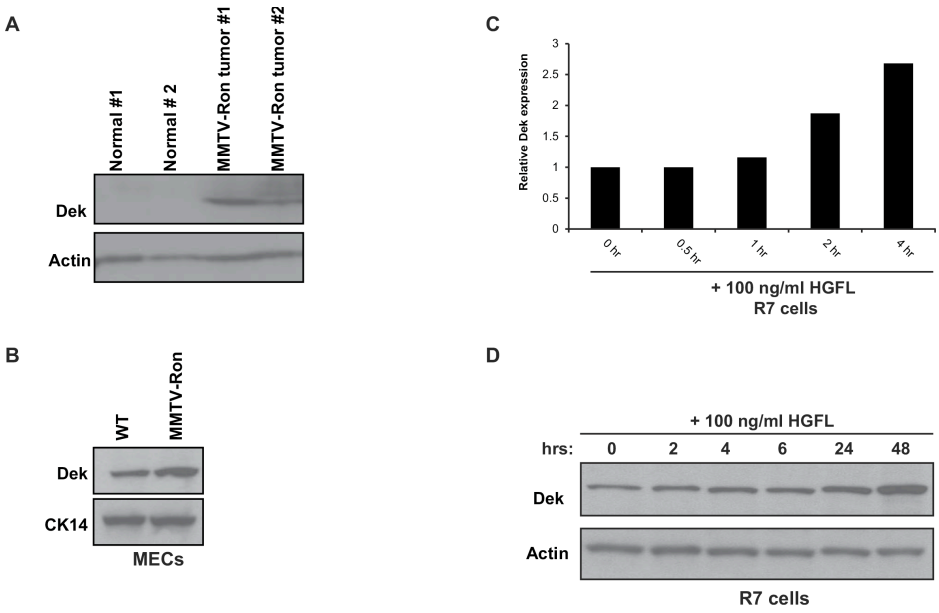

Figure 1. DEK expression is induced following Ron overexpression and activation. A. Western blot analysis for DEK protein levels from two independent normal mammary glands and from two tumor bearing glands from MMTV-Ron mice. **B.** DEK protein expression examined by Western blot analysis on purified mammary epithelial cells (MECs) from wild type (WT) and MMTV-Ron mice. Cytokeritin 14 (CK14), an epithelial cell marker, is used as a control. **C.** Relative Dek mRNA expression in a MMTV-Ron derived mammary tumor cell line (R7 cells) treated with the Ron ligand, HGFL. **D.** Dek proteins levels in R7 cells following HGFL treatment temporally.

To directly assess the functional requirement for Dek on mammary tumorigenesis downstream of Ron activation, we bred MMTV-Ron mice with mice deficient in Dek (Dek-/- mice) to generate MMTV-Ron mice proficient (Dek+/+) and deficient (Dek-/-) for Dek. Female mice were monitored temporally for palpable mammary tumor development. As depicted in **Figure 2A**, MMTV-Ron mice deficient in Dek exhibited a significantly reduced rate of tumor initiation compared to Dek wild-type mice. Tumor kinetics in Dek+/- mice were intermediate between Dek+/+ and Dek -/- mice (data not shown). The average time to tumor initiation in MMTV-Ron Dek+/+ mice was 202 days compared 230.5 days for MMTV-Ron Dek-/- mice. These data represent the first to demonstrate a genetic requirement for Dek in mediating mammary tumorigenesis and provide concrete evidence for an oncogenic function of Dek in breast tumor formation. While tumor initiation was delayed, mice of both genotypes exhibited 100% penetrance of tumor formation and tumors that formed were histologically similar. In examining pre-neoplastic glands between genotypes, Dek expression was found to be important to maintain epithelial cell proliferation with a 2-fold reduction in BrdU staining observed in Dek-/- glands compared to controls (**Figure 2B**).

To examine phenotypic and biochemical signaling events on Dek expression, mammary tumor cell lines were generated dependent from MMTV-Ron Dek+/+ and MMTV-Ron Dek-/- mice. Several tumor derived cell lines were generated. As depicted in **Figure 3**, three MMTV- Ron Dek-/- cell lines were generated (RD147R, RD238, and RD271) and either transduced with a control vector (R780) or with a vector to re-express mouse Dek (R780:mDek). Similarly, two cell lines were generated from MMTV-Ron Dek+/+ mammary tumors (RD272N and RD258N) and were transduced with a control vector (NTsh) or a Dek targeting shRNA (Deksh2). Cells were subsequently tested *ex vivo* for growth over time (**Figure 3A**) and in migration assays (**Figure 3B**). As shown in **Figure 3**, Dek is required for optimal mammary tumor cell proliferation, and Dek reconstitution lead to significant increases in tumor cell migration, further supporting the alterations in tumor kinetics observed in **Figure 2**.

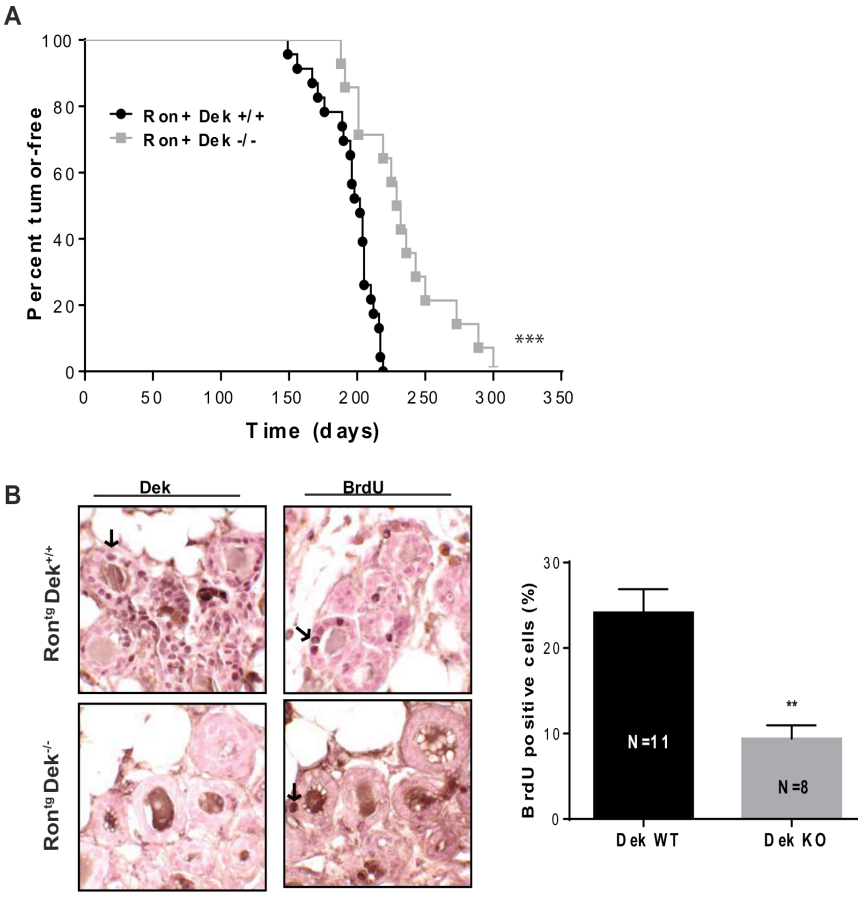

Figure 2. Dek loss delays tumor initiation and the growth of mammary hyperplastic foci. A. Tumor kinetics in MMTV-Ron (Ron+) mice proficient (Dek+/+) and deficient (Dek-/-) in Dek. **B**. Immunohistochemial staining of tumor foci in Ron+ Dek+/+ and Ron+ Dek-/- mammary glands. Staining was performed for Dek expression and BrdU incorporation. Arrows denote positive staining and the histogram represents the percentage of BrdU positive epithelial cells per field. "N" denotes the number of mice analyzed.

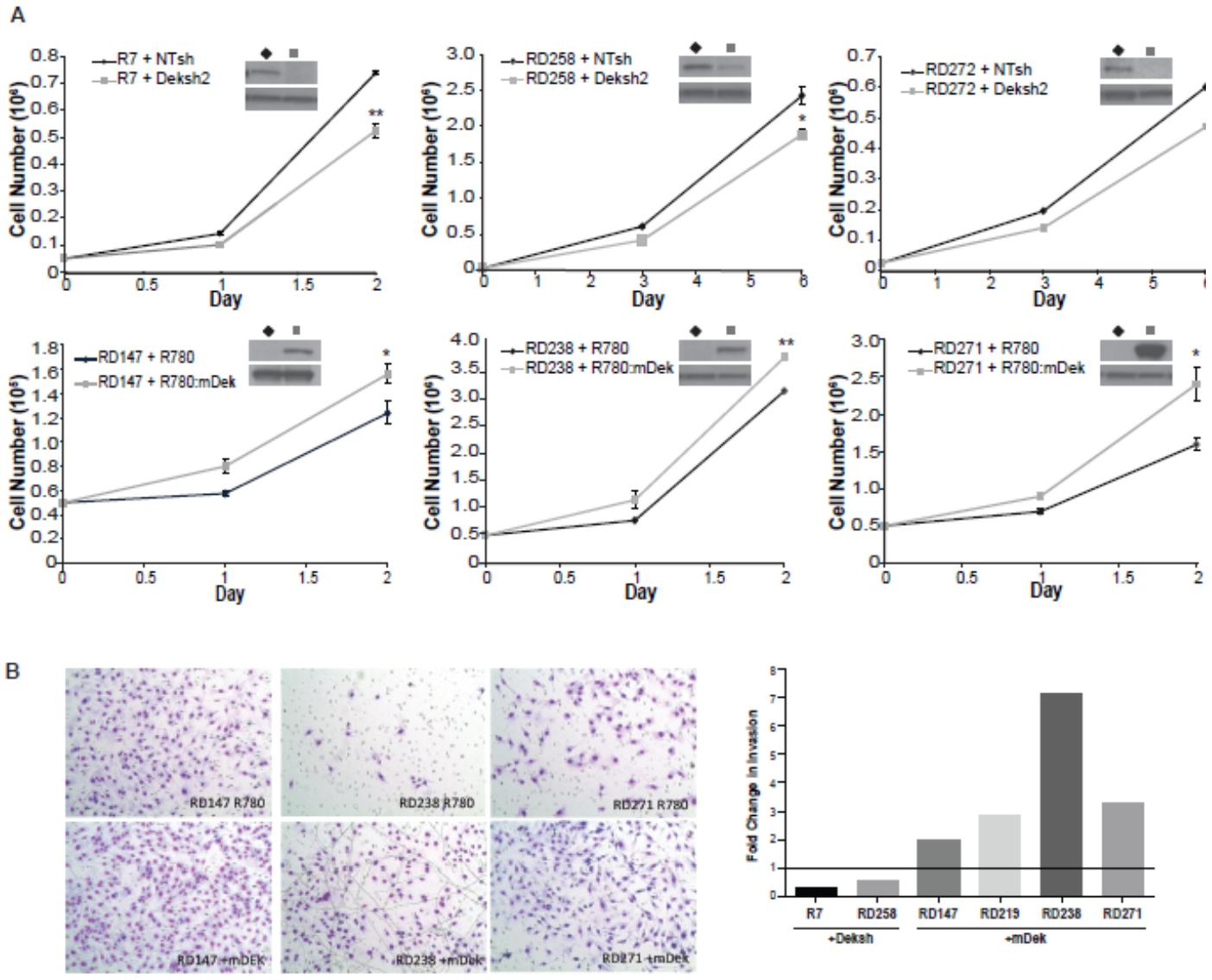

Figure 3. Dek loss inhibits mammary tumor cell growth and proliferation. A. Mammary tumor cell lines from MMTV-Ron mice were generated and either reconstituted with Dek or transduced with a Dek knockdown construct. Cells were plated and cell counts were examined over the time frame indicated. Grey bars refer to Dek loss while the corresponding black bars relate to Dek expressing cells. In each case, Dek deficiency reduced tumor cell growth. **B.** Cells from A were plated in Matrigel invasion chambers and tumor cell migration to serum was examined. Depicted are representative fields of stained cells which have successfully migrated after 22 hours. Of note, Dek expression augments the migratory ability of these cells. Quantification of cell invasion changes are quantified on the right and are represented as fold change compared to empty vector controls for each cell line. Dek depletion (left two cell lines) decreases invasion while Dek complementation in Dek-/- cell lines enhances invasive phenotypes.

Given the connection of both Ron and Dek with activation of beta-catenin (1-4), we next sought to establish this link by examining the Wnt signaling pathway through RT-qPCR and western blot analysis of Dek-proficient and Dek-depleted mammary tumor cells lines. As shown in **Figure 4A**, we made the novel connection of Dek with alterations in Wnt ligands. Message levels for multiple Wnts were repressed in DEK knockdown when compared to control cells, and induced in DEK reconstituted cells when compared to control cells. Particularly strong induction was observed for Wnt4 and Wnt10b following DEK reconstitution. Wnt10b regulation was additionally supported by western blot analysis (**Figure 4B**). To further validate this data *in vivo*, a Dek expressing mammary tumor cell line (R7) from MMTV-Ron mice was knocked down for Dek expression (R7 Deksh2). Dek expressing control (R7 NTsh) and depleted cells (R7 Deksh2) were orthotopically injected in the mammary fat pads of nude mice and tumor growth was determined over time (**Figure 4C**). Dek depletion resulted in a significant inhibition of tumor growth. In addition, mammary tumor sections from each group were stained for both Dek and for Wnt10b by immunohistochemistry (IHC). IHC data confirmed Dek depletion in the

R7 Deksh tumors compared to controls and also a substantial decrease in Wnt 10b detection. To further extend this data, we also examined the RD147 mammary tumor cell line (deficient in Dek) along with the reconstituted line for beta-catenin activation by reporter assays. As depicted in **Figure 4D**, Dek expression dramatically promoted beta-catenin dependent reporter activity. In total, our data provide the first genetic evidence for the significance of Dek *in vivo* on mammary tumorigenesis and suggest that Dek controls tumor cell proliferation and migration though a Wnt-dependent mechanism. In the next funding cycle, we intend to define the extent of metastatic dissemination in tumor models as well as elucidate the mechanism by which Dek promotes beta-catenin activation downstream from Ron.

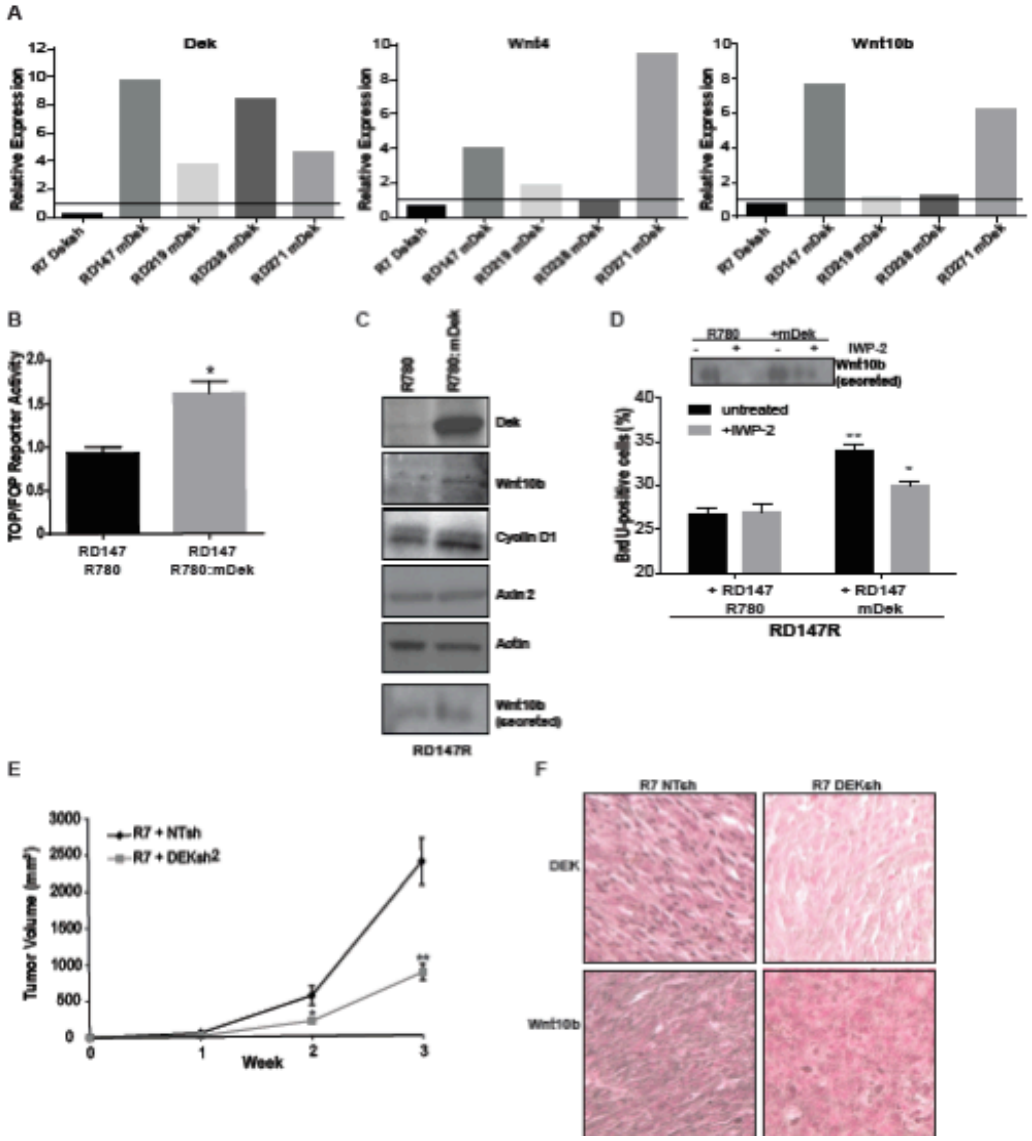

Figure 4. Dek expression regulates Wnt ligand production and beta-catenin activation during breast tumorigenesis. A. Quantitative RT-PCR was performed on R7 (Dek+/+) cells with DEKsh and four Dek-/- cell lines complemented with murine Dek (mDek; Dek levels in left panel). Wnt4 (center) and Wnt10b (right) were consistently upregulated in Dek expressing cells and downregulated in Dek depleted cells. Graphs depict fold changes compared to empty vector controls for each cell line. **B.** TOP/FOP luciferase reporter assays for beta-catenin activity show that Dek complementation in RD147 enhances transcription from a TCF/LEF/beta-catenin reporter. **C.** Western blotting of RD147R Dek-/- cells complemented for murine Dek show increased levels of Wnt10b, increased cyclin D1 as a marker of proliferation, and a 2-fold increase in secreted Wnt10b (bottom). **D.** Dek expression induces the proliferation of neighboring cells in a Wnt-dependent manner. Flow cytometry was performed to detect BrdU as a marker of proliferation in GFP- parental RD147 cells that were co-cultured with GFP+ RD147 R780 or R780:mDek. IWP-2 is a chemical inhibitor of Porcupine that prevents Wnt ligand secretion. The inset western blot depicts levels of secreted Wnt10b in conditioned media in untreated and IWP-2 treated cells. Incomplete rescue of proliferative changes may be due to residual Wnt10b secretion in R780:mDek cells. **E.** Dek depletion in R7 cells decreases tumor growth in orthotopic xenografts (left) and decreases Wnt10b levels as detected by immunohistochemistry (right; **F**)

The first objective of Task 2 was to test DEK-targeting 1716 Herpes Simplex Viruses (1716HSV) on breast cancer cells. A panel of vectors was constructed by our collaborators at Virttu Biologic, and these were designed to silence DEK expression and selectively replicate in tumor tissue. They had shown promise in primary squamous cell carcinoma cells. However, our initial experiments using breast cancer cells raised concerns about the degree of knockdown that could be achieved. Three DEK targeting viruses were tested for DEK knockdown in HeLa cells. These included GFP-expressing viruses with 2 or 4 tandem short RNA hairpins subcloned from the pLKO DEKsh2 lentiviral vector. Previously, we had shown that pLKO DEKsh2 achieves >90% DEK transcript silencing in breast cancer cells (5), as well as in HeLa cells (data not shown), and were expecting similar knockdown efficiency in 1716HSV based on these and our preliminary data in primary squamous cell carcinoma cells. An additional experimental antisense vector was provided by Virttu Biologics to be compared against a GFP-tagged control parent virus (**Figure 5A**). The vectors were tested side-by-side with our established adenoviral DEK knockdown system (AdDEKsh) (6). HeLa cells were infected with 10 virus particles per cell (10 multiplicity of infection, MOI), and infection efficiency was assessed by GFP expression at 14 hours post-infection (**Figures 5B, 5D**). Following harvest at 15 hours, the control adenoviral system demonstrated robust transcript silencing by RT-PCR (**Figure 5C**), but the 1716DEKsh or antisense viruses were unable to suppress DEK transcript levels (**Figure 5E**). Similar results were obtained in 1716HSV replication-resistant cell types harvested at late time points (**Figure 6A-B**), MDA MB 231 breast cancer cells treated with a HSV DNA polymerase inhibitor (**Figure 6C**), and by protein expression in human foreskin keratinocytes (**Figure 6D**). Taken together, the data suggest that the shRNA construct fails to express or is not properly processed for recognition by the DICER system. Virttu Biologics is considering all possibilities and modification of the next generation of DEK-targeting oncolytic vectors.

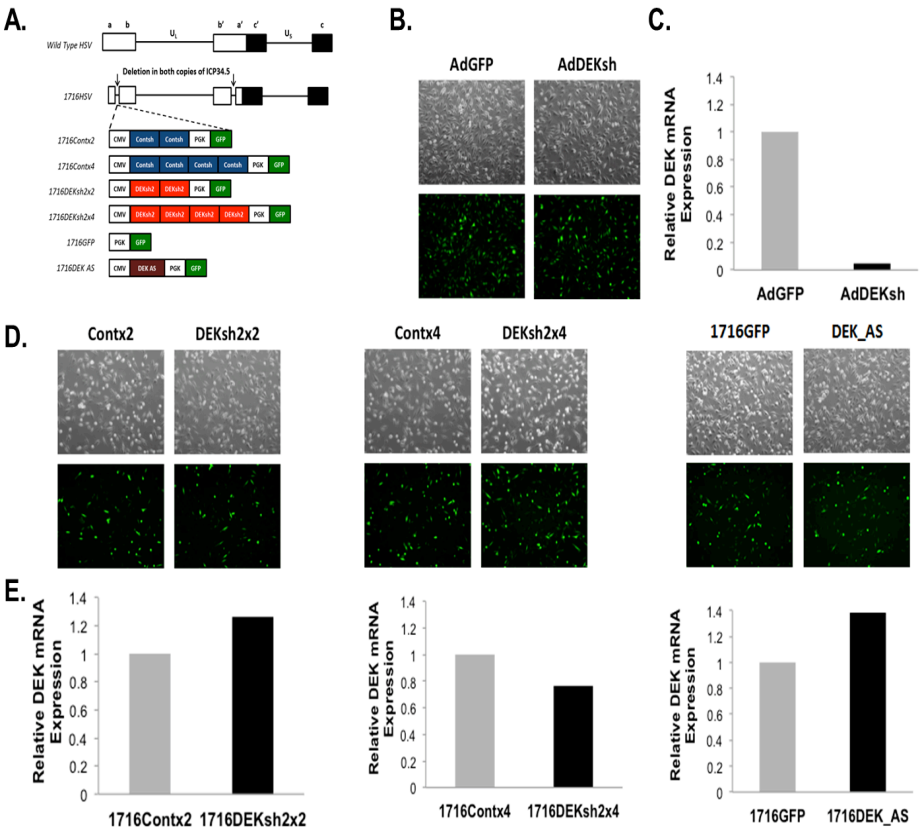

Figure 5: HeLa cells were infected with 10 MOI of HSV or control adenovirus and harvested at 15 hours for DEK mRNA expression. **(A)** Schematic of 1716DEKsh and 1716Cont control viruses. Tandem shRNA cassettes were driven by a CMV promoter. **(B)** Control HeLa were infected with 10 MOI of adenovirus equipped with GFP (AdGFP) or GFP and DEKsh cassettes (AdDEKsh). Representative image of GFP 14 hours following infection. **(C)** qRT-PCR harvested from (B). **(D)** Representative images of GFP expression following infection with 10 MOI of control and knockdown virus pairs. **(E)** RNA was harvested from (**D**) and DEK transcript quantified by qRT-PCR.

Concurrent with the shRNA function validation experiments, virus-mediated cytotoxicity was determined via the MTS assay. The DEKsh2x4 (**Figure 7A**) or DEKsh2x2 (not shown) cassettes did not improve cytotoxicity over control virus. Since the DEK knockdown construct did not affect 1716HSV cell killing as a monotherapy, these viruses were tested as an adjuvant to sensitize the UMSCC1 head and neck carcinoma line to cisplatin therapy (**Figure 7B**). While both control and DEK knockdown viruses enhanced cisplatin cytotoxicity, the knockdown cassette did not further improve cytotoxicity at any MOI (**Figure 7C-E**). These findings are in line with the promise of combining virotherapy and chemotherapy, and we expect that HSV constructs capable of targeting DEK will be more efficient over the parental vector.

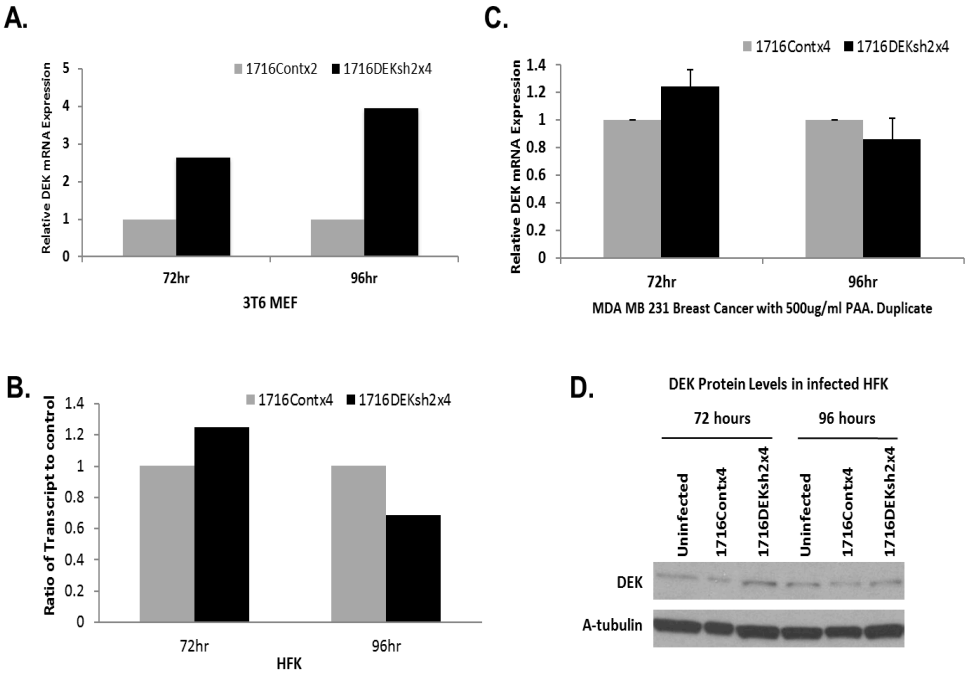

Figure 6: DEK knockdown following medium term 1716DEKsh infection were tested in HSV replication resistant cells and breast cancer lines. **(A)** 3T6 MEF cells resistant to 1716HSV replication, were infected with 1.25 MOI and harvested at 72 and 96 hours post-infection for DEK transcript quantification. **(B)** Primary human foreskin keratinocytes (HFKs) which are relatively resistant to 1716HSV replication, were infected with 0.06 MOI of virus and were harvested for mRNA at 72 and 96 hours. **(C)** MDA MB 231 breast cancer lines were infected with 1 MOI of virus, and viral replication was inhibited using 500ug/ml phosphonoactic acid. **(D)** HFK were infected as in (B) and were harvested for protein at 72 and 96 hours.

Taken together, preliminary characterization of the 1716HSV DEK knockdown vectors revealed these constructs are unable to efficiently target DEK, perhaps due to poor shRNA expression or processing. It is likely that the lack of functional differences in the cytotoxicity assays reflects the observed lack of DEK knockdown. We are continuing to work with Virttu Biologics to design the next generation of 1716DEKsh constructs that will robustly silence DEK expression. The 1716HSV platform remains the most viable strategy to therapeutically target the DEK oncogene as the protein is not yet amenable to small molecule treatment and a crystal structure not available. We look forward to testing the next generation of viruses as soon as they arrive, and all required assays are now in place for this purpose.

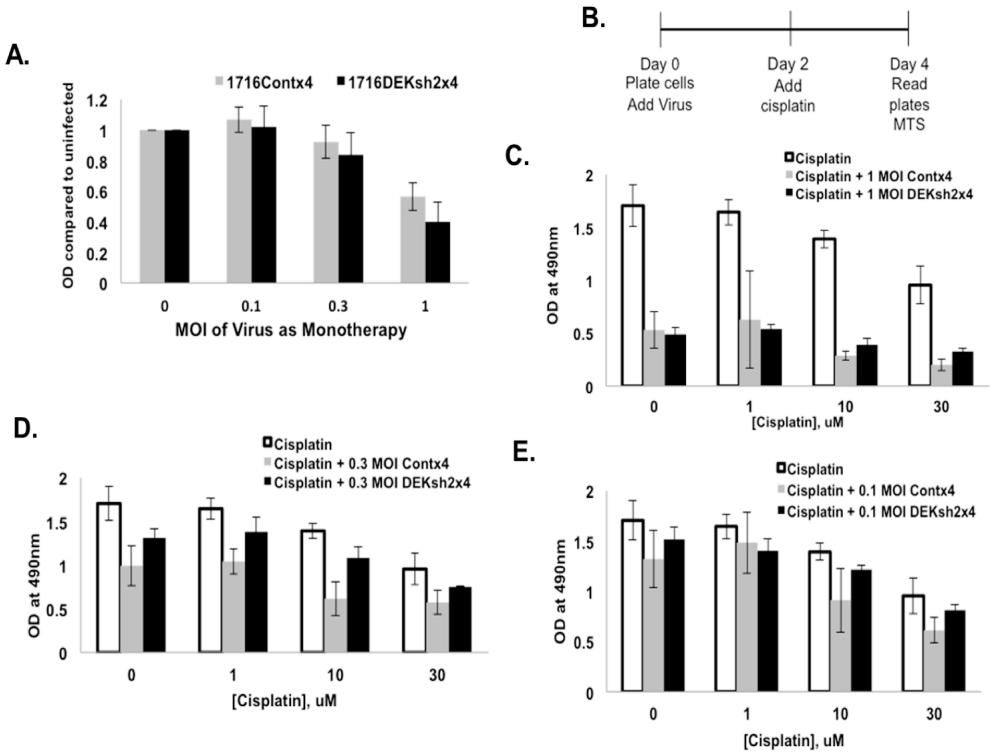

Figure 7: As a monotherapy or in combination with cisplatin, 1716DEKsh2x4 does not display enhanced cytotoxicity compared to 1716Contx2. **(A)** Virus monotherapy cytotoxicity in UMSCC1 head and neck carcinoma cells was determined by three separate MTS experiments, each with n=5 data points. Readings were taken at day 4 post-infection. **(B)** Virus and cisplatin were added at the indicated times for subfigures C-E. Following infection with 1 MOI **(C)**, 0.3 MOI **(D)**, or 0.1 MOI **(E)** of 1716DEKsh2x4 or 1716Contx4, UMSCC1 were treated with cisplatin and cytotoxicity was assessed by MTS.

KEY RESEARCH ACCOMPLISHMENTS

- Staffing in place to support continuation of the project
- Generated compound mice containing mammary specific Ron overexpression (MMTV-Ron) combined with DEK hetero and homozygosity.
- Initiated procedures to successfully genotype mice from breeding colonies.
- Defined the impact of Dek loss in Ron overexpressing mammary tumors though temporal analysis of tumor kinetics, incidence, and growth.
- Identified by western blot analysis, immunohistochemistry and qRT-PCR the alteration in Wnt ligand induction and in beta-catenin activation in Dek proficient and depleted cells.
- Performed initial testing of

REPORTABLE OUTCOMES

Gene Targeted Animals:
Generated breeding colonies to obtain MMTV-Ron mice that are wild type (+/+), heterozygous (+/-) and deficient (-/-) for Dek.

Cell lines:
Generated several mammary tumor cell lines from MMTV-Ron mice with and without Dek deficiency.

Research Opportunities:

Sasha Ruiz, Graduate student, 2013-present
Eric Smith, PSTP Graduate student, 2012-present
Purnima Wagh, PhD, 2012-2013
Nancy Benight, PhD, 2012-present
Juana Serrano-Lopez, visiting scientist, 2013-present
Nicholas Pease, research assistant, 2013-present
Lisa Privette, PhD, 2013-present

Manuscripts under preparation:
Privette Vinnedge, L.M., Wagh, P.K. Benight, N., Serrano-Lopez, J., Cancelas, J., Waltz, S.E., and Wells, S.I., "The DEK oncogene promotes cell proliferation through paracrine Wnt signaling in Ron receptor positive breast cancers," *in preparation*

Platform presentations at national meetings:
Privette Vinnedge, L.M., Wagh, P.K., Serrano-Lopez, J., Waltz, S.E., Wells, S.I. "The Dek Oncogene Drives Breast Cancer Progression and Chemotherapeutic Resistance," Tenth Anniversary Interdisciplinary Women's Health Research Symposium, National Institutes of Health, Bethesda, MD, October 24, 2013

CONCLUSIONS

- Our data provide the first direct demonstration that the oncogene Dek is a downstream target of Ron receptor signaling in breast cancer.
- The Dek upregulation observed in Ron expressing breast tumors provides a growth and migratory/invasive phenotype to the breast cancer cells.
- Dek loss in MMTV-Ron mice significantly reduces the time to tumor imitation and is associated with decreased breast cancer cell proliferation.
- Dek expression controls key molecules involved in Wnt signaling in breast tumors and in breast cancer cell lines.
- Oncolytic virsus containing a shRNA for Dek was produced.
- Initial testing of the shDek oncolytic virus has shown that the shDek oncolytic virus is similarily effective compared to the oncolytic virus alone. Further testing has shown that the shRNA is not effectively knockdown Dek.
- The next generation of Dek targeting oncolytic vectors is being planned.

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APPENDICES

None